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Flavin adenine dinucleotide as a chromophore of the *Xenopus* (6–4)photolyase

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ABSTRACT

Two types of enzyme utilizing light from the blue and near-UV spectral range (320–520 nm) are known to have related primary structures: DNA photolyase, which repairs UV-induced DNA damage in a light-dependent manner, and the blue light photoreceptor of plants, which mediates light-dependent regulation of seedling development. Cyclobutane pyrimidine dimers (CPDs) and pyrimidine (6–4) pyrimidone photoproducts [(6–4)photoproducts] are the two major photoproducts produced in DNA by UV irradiation. Two types of photolyases have been identified, one specific for CPDs (CPD photolyase) and another specific for (6–4)photoproducts [(6–4)photolyase]. (6–4)Photolyase activity was first found in *Drosophila melanogaster* and to date this gene has been cloned only from this organism. The deduced amino acid sequence of the cloned gene shows that (6–4)photolyase is a member of the CPD photolyase/blue light photoreceptor family. Both CPD photolyase and blue light photoreceptor are flavoproteins and bound flavin adenine dinucleotides (FADs) are essential for their catalytic activity. Here we report isolation of a *Xenopus laevis* (6–4)photolyase gene and show that the (6–4)photolyase binds non-covalently to stoichiometric amounts of FAD. This is the first indication of FAD as the chromophore of (6–4)photolyase.

INTRODUCTION

Light is essential for life on Earth and organisms have evolved various method for efficient utilization of light energy. Within the spectrum of sunlight, near-UV/blue light (320–520 nm) is utilized very efficiently and elegantly by two related systems. (i) In contrast to the many beneficial effects of solar light, the UV component is harmful to living cells, producing cytotoxic, mutagenic and carcinogenic lesions in DNA (1–3). This DNA damage can be repaired by near-UV/blue light by the DNA repair enzyme DNA photolyase (4,5). (ii) Numerous environmental factors influence plant development. Of these, light has an especially important role as a stimulus for many developmental processes. Blue light markedly affects growth and development of higher plants, including such phenomena as phototropism, chloroplast rearrangement, stomatal opening and inhibition of hypocotyl elongation. These responses are mediated by a blue light photoreceptor, cryptochrome (6).

The phenomenon of photoreactivation, the reduction of the lethal and mutagenic effects of UV radiation by simultaneous or subsequent irradiation with near-UV/blue light, has been identified in a variety of organisms. The enzyme responsible, CPD photolyase, binds to UV-damaged DNA and on absorption of a near-UV/blue light photon splits the cyclobutane ring, restoring the bases to their native form (7). In this reaction, the near-UV/blue light photon is used to excite $FADH^-$ and flavin in the excited state then donates an electron to the CPD and thus FAD is essential for the reaction. The CPD photolyase gene has been isolated from 13 organisms and, on the basis of deduced amino acid sequence similarities, the genes have been grouped into two classes: Class I and Class II (8,9).

Light-dependent plant development, a complex process called photomorphogenesis, is controlled by the combined action of several photoreceptor systems (10). In higher plants there are at least three different families of photoreceptors: the red/far-red light receptor (phytochromes), the blue light receptor (cryptochrome; CRY) and a receptor for UV light. Although the best-studied signaling pathway in plants involves phytochrome, considerable research has been carried out in the past decade to characterize blue light perception and the signal transduction pathway (6). Recently, the first blue light photoreceptor in plants was characterized at the molecular level (11). This protein (CRY1) shows close homology to Class I CPD photolyase, although it exhibits no photolyase activity. CRY1 also binds FAD (12), suggesting that CRY1 mediates a light-dependent redox reaction similar to CPD photolyase.

Recently, we discovered another type of photolyase in *Drosophila melanogaster* that catalyzes the light-dependent repair of (6–4)photoproducts instead of CPDs and named this molecule

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(6–4)photolyase (13). Subsequently, the same enzymatic activity was identified in *Xenopus laevis*, *Crotalus atrox* (14) and *Arabidopsis thaliana* (15). It was previously thought that photoenzymatic reversal of (6–4)photoproducts was very unlikely for the following reasons. The formation of (6–4)photoproducts involves the transfer of the group at the C-4 position (-NH or -OH) of the 3′ base of the dinucleotide to the C-5 position of the 5′ base concomitant with the formation of a sigma bond between the C-6 of the 5′ base and the C-4 of the 3′ base. Even if an enzyme breaks the sigma bond joining the two adjacent pyrimidines, the bases would not be restored to their original forms. Thus, the mechanism of photoreactivation of (6–4)photoproduct is different from that of CPD (16). The gene encoding (6–4)photolyase was cloned from *Drosophila* (17). Unexpectedly, the deduced amino acid sequence of (6–4)photolyase was found to be similar to the Class I CPD photolyase and CRY1. Thus we call these proteins the DNA photolyase/blue light photoreceptor family. Based on the amino acid sequence similarity, we set out to clone the *Xenopus* (6–4)photolyase cDNA by polymerase chain reaction (PCR). Here, we describe the isolation and characterization of a cDNA encoding (6–4)photolyase from *X.laevis*. We show that (6–4)photolyase binds FAD similarly to other member of the DNA photolyase/blue light photoreceptor family, although CPD and (6–4)photolyase operate by different mechanisms.

MATERIALS AND METHODS

Preparation of crude cell extracts from *Xenopus* ovaries

Isolated ovaries were homogenized in 1 ml buffer containing 10 mM Tris–HCl, pH 8.0, 1 mM EDTA, 5 mM dithiothreitol (DTT) and 0.5 mM phenylmethylsulfonyl fluoride (PMSF). To the homogenate was added 1 ml ice-cold solution containing 10 mM Tris–HCl, pH 8.0, 5 mM DTT, 25% sucrose and 50% glycerol. After mixing gently, 160 µl 5 M NaCl was added, mixed for 20 min at 4°C and then centrifuged at 15 000 *g* for 20 min at 4°C. The supernatant was used as crude extract. Aliquots of 2 µg crude extract were used for gel shift assays as described previously (13,18).

Isolation of *Xenopus* (6–4)photolyase cDNA clone

Unless otherwise noted, all DNA and RNA manipulations were carried out using standard techniques (19). To prepare the probe for hybridization, PCR was carried out with *Xenopus* ovary cDNA and degenerate oligonucleotides (64PRN1, 5′-T[A/G/C/T]GC[A/G/C/T]TGG[A/C]G[A/G/C/T]GA[A/G]TT[T/C]-TA-3′; 64PRC1, 5′-CC[T/C]TC[T/C]TCCCA[A/G/C/T][G/C][A/T][A/G/T]ATCCA-3′; 64PRC2, 5′-TG[A/G/C/T]C[G/T]GGC[A/G/C/T]AG[A/G]TG[A/G]TG[A/G/C/T]ATCCA-3′) based on regions conserved between CPD photolyase and (6–4)photolyase (17). Two rounds of PCR were carried out. The first round was carried out with primers 64PRN1 and 64PRC1 and aliquots of the first round PCR product were used for the second round of PCR with primers 64PRN1 and 64PRC2. A 160 bp amplified product was sequenced and found to be related to *Drosophila* (6–4)photolyase and this was used to screen a *Xenopus* oocyte cDNA library (Clontech). Four positive clones were isolated, the longest of which was recloned into pUC19 and sequenced on both strands by the standard dideoxy chain termination method (19).

Purification of *Xenopus* (6–4)photolyase

Plasmid pGEX-Xl64PR was constructed by inserting the coding sequence of *Xenopus* (6–4)photolyase cDNA into *Bam*HI/*Eco*RI-digested pGEX-4T-2 (Pharmacia) and used for transformation of *Escherichia coli* SY2(*uvrA*$^-$, *recA*$^-$, *phr*$^-$) (20). Transformed cells were grown at 26°C in 3 l LB medium containing 150 mg/l ampicilin (19) until an A_{600} of 0.9–1.0 was reached. Expression was induced by addition of 0.1 mM isopropyl β-D-thiogalactopyranoside (IPTG) and growth was continued at 26°C for 9 h. Cells were harvested by centrifugation and resuspended in 50 ml phosphate-buffered saline (PBS). Cell extract was prepared by sonication of the cell suspension, followed by centrifugation at 15 000 *g* for 60 min at 4°C. The supernatant (Fraction I) was applied to a glutathione–Sepharose column (10 ml). Purification using glutathione–Sepharose and removal of glutathione S-transferase (GST) by cleavage with thrombin were performed according to the manufacture's instructions (Pharmacia). The eluate from the glutathione–Sepharose column (Fraction II) was treated with thrombin and the thrombin-cleaved sample (Fraction III) was applied to a UV-irradiated DNA affinity column equilibrated with 50 mM phosphate buffer, pH 7.5, containing 50 mM KCl. After washing with 15 ml equilibration buffer, bound protein was eluted with 15 ml elution buffer (50 mM phosphate buffer containing 2 M KCl). The eluted sample was concentrated using Centriprep 50 (Amicon) and elution buffer was replaced with equilibration buffer. Finally, 1 ml protein solution was obtained (Fraction IV). Starting from 3 l *E.coli* culture, 700 mg cell extract (Fraction I), 5 mg protein eluate from glutathione–Sepharose (Fraction II) and 1.4 mg UV-irradiated DNA affinity column purified protein (Fraction IV) were recovered. The concentration of protein was determined with a Bradford assay kit (BioRad). The UV-irradiated DNA affinity column was prepared as described previously (21). Photoreactivation treatment and ELISA were carried out as described previously (13,17). For ELISA and for the repair assay using a (6–4)photoproduct-containing oligonucleotide, 0.1 and 1 µg Fraction III were used respectively.

Preparation of a DNA fragment containing (6–4)photoproduct

The deoxyoligonucleotide 28mer substrate [d(CCCGAACAGA-CAGT[6–4]TAACCACGCAAACG)] containing a (6–4)photoproduct at the central TT site was constructed by ligation of a (6–4)photoproduct-containing 8mer [d(CAGT[6–4]TAAC)] with a 10mer [d(CCCGAACAGA) and d(CACGCAAACG] after annealing with a 32mer [d(TTCGTTTGCGTGGTTAACTGTC-TGTTCGGGTT)] using the procedure described previously (22). Resultant duplex DNA was purified by gel electrophoresis and labelled with [γ-^{32}P]ATP (3000 Ci/m mol) and T4 polynucleotide kinase. The labelled DNA (5×10^4 c.p.m.) was mixed with purified *Xenopus* recombinant protein (1 µg Fraction III) and exposed to fluorescent lamps for 30 min. After irradiation the DNA was then extracted with phenol/chroloform and precipitated with ethanol. The DNA was digested with *Hpa*I (10 U) and separated on 10% polyacrylamide sequencing gels.

Chromophore isolation from recombinant *Xenopus* (6–4)photolyase and reconstitution of *E.coli* CPD photolyase

Recombinant *Xenopus* (6–4)photolyase purified with a UV-irradiated DNA affinity column (Fraction IV) was denatured at pH 3.0 by heating at 65°C for 10 min. The released chromophore was recovered by filtering out the denatured protein using Microcon 30 followed by Microcon 3 (Amicon). *Escherichia coli* photolyase apoenzyme was prepared as described previously (23). Reconstitu-

tion of enzymatic activity with either authentic FAD or chromophore isolated from *Xenopus* (6–4)photolyase was conducted by incubating the apoenzyme (400 μM) with the indicated chromophore (40 μM) at 10°C for 24 h (24). The concentration of chromophore was based on the absorbance at 450 nm (ε_{450} = 1.12 × 10^4 M^{-1} cm^{-1}).

CPD photoreactivation assay

The oligo(dT)$_{20}$ substrate containing CPDs was prepared by acetone-photosensitized irradiation under a cold nitrogen atmosphere. Since CPD has no absorption at 265 nm, the increase in absorbance at 265 nm was used to estimate CPD repair (25). For the photoreactivation assay, enzyme (4 μM) was mixed with substrate (30 μM) in 200 μl buffer containing 50 mM Tris–HCl, pH 7.4, 50 mM NaCl, 1 mM EDTA, 5 mM DTT and 10% glycerol. The reaction mixture was placed in a cuvette, deoxygenated under a gentle stream of cold nitrogen and exposed to filtered camera flashes (340 nm cut-off filter) prior to irradiation with photoreactivating light (350–450 nm) at 10°C.

RESULTS

Cloning of *Xenopus* (6–4)photolyase

Previously we have shown that in *Drosophila* DNA photolyase genes are expressed at a very high level in the ovary and its translated products are stored in eggs (9,17). This suggests that ovary is a good candidate for testing (6–4)photolyase activity to screen mRNA in *X.laevis*. We tested binding activity specific for (6–4)photoproduct in cell extracts from *Xenopus* ovaries using the same gel shift assay as reported previously (17). We detected a factor which binds specifically to (6–4)photoproduct in *Xenopus* ovary cell extracts (Fig. 1). To generate a probe to screen a *Xenopus* cDNA library, we used PCR with primers based on regions conserved between the DNA photolyase/blue light photoreceptor family (see Materials and Methods). A 160 bp DNA fragment amplified from cDNA prepared from *Xenopus* ovaries was used as a probe to screen a *Xenopus* oocyte cDNA library and we identified a 2.5 kb cDNA clone. Sequencing of the cDNA clone revealed the presence of a single long open reading frame capable of encoding a protein of 526 amino acids, corresponding to a predicted molecular mass of 60.6 kDa. The sequence of the cDNA predicted a protein that showed 58–54% amino acid identity to *Drosophila* (6–4)photolyase and its human homologue and 20–24% identity to the class I CPD photolyase and the blue light photoreceptor over the entire protein (data not shown). Thus the cloned cDNA is a member of the DNA photolyase/blue light photoreceptor family.

Expression of the cDNA in *E.coli*

To verify that the isolated cDNA clone encodes (6–4)photolyase, we measured enzymatic activity of the recombinant protein expressed in *E.coli*. The cDNA was inserted into a prokaryotic expression vector designed to produce a GST fusion protein and named pGEX-Xl64PR. *Escherichia coli* does not photoreactivate (6–4)photoproduct and thus would show increased resistance to UV light on expression of (6–4)photolyase in the presence of photoreactivating light. As expected, the plasmid pGEX-Xl64PR conferred light-dependent UV resistance on *recA*$^-$*uvrA*$^-$*phr*$^+$ *E.coli* (Fig. 2A). The recombinant protein was purified from *E.coli* cell extract as a single 60 kDa band on SDS–PAGE (Fig. 2B).

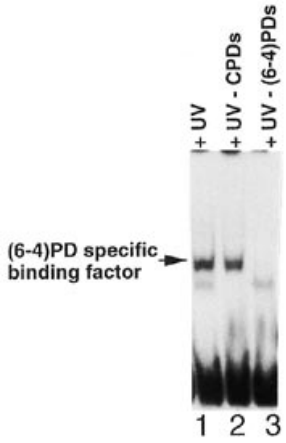

Figure 1. Gel shift analysis showing a binding factor from *Xenopus* ovary cells that has high affinity for (6–4)photoproduct. Crude extract was examined by gel shift assay for binding activity toward a UV-irradiated TC-3 DNA probe (17). TC-3 DNA was irradiated with 25 kJ/m^2 UV and used directly (lane 1) or after treatment with *E.coli* CPD photolyase to deplete CPDs (lane 2) or with *Drosophila* (6–4)photolyase to deplete (6–4)photoproduct [(6–4)PD] (lane 3). The arrow indicates the shifted band formed with (6–4)photoproduct-specific binding factor.

Its absorption spectrum indicated that the purified protein eluted from the UV-irradiated DNA affinity column did not contain a second chromophore and possessed fully oxidized FAD (see below). Thus, the thrombin-cleaved glutathione–Sepharose eluate (Fraction III) was used for determination of (6–4)photolyase activity. Enzyme-linked immunosorbent assay (ELISA) showed that the purified recombinant protein eliminated (6–4)photoproduct from UV-irradiated DNA in a light-dependent manner, although it had no effect on CPDs (Fig. 2C). Furthermore, the recombinant protein repaired (6–4)photoproduct, as shown in Figure 2D. A 32 bp DNA containing a (6–4)photoproduct at a TT sequence in the *Hpa*I site (5′-TTAA-3′) was resistant to digestion with *Hpa*I, whereas it became *Hpa*I-sensitive after photoreactivation with the purified recombinant protein. Together these results show that the cDNA clone in pGEX-Xl64PR encodes the (6–4)photolyase.

Identification of the chromophore

Purified *Xenopus* (6–4)photolyase was a yellow colour and had an absorption spectrum resembling those of many flavoproteins (Fig. 3). The chromophore was released by heat or acid treatment of *Xenopus* (6–4)photolyase, indicating that it was non-covalently bound to the enzyme. The absorption spectrum of the free chromophore was identical to that of fully oxidized flavin adenine dinucleotide (FAD) (Fig. 3). The identity of the chromophore as FAD was also suggested by thin layer chromatography and an increase in fluorescence intensity on acidification (data not shown). To determine that this chromophore was indeed FAD, we reconstituted *E.coli* CPD photolyase activity from its apoenzyme and the chromophore isolated from *Xenopus* (6–4)photolyase. *Escherichia coli* CPD photolyase requires bound FAD as a catalytic cofactor. The holoenzyme (FAD–bound *E.coli* photolyase) showed high affinity for CPDs (Fig. 4A, lane 5) and repaired them in a light-dependent manner, although the apoenzyme had no affinity for CPDs (Fig. 4A, lane 2) and no photocatalytic activity (Fig. 4B) (23). When the apoenzyme was mixed with the chromophore isolated from *Xenopus* (6–4)photolyase, the resulting reconstituted photolyase restored both binding (Fig. 4A, lane 3)

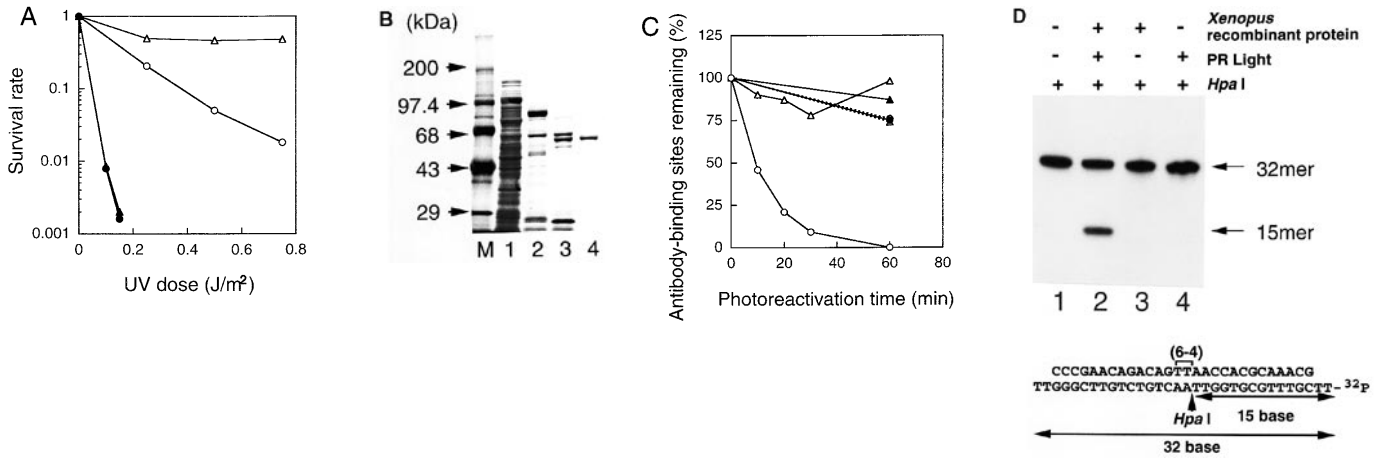

Figure 2. Photoreactivating activity of *Xenopus* (6–4)photolyase expressed in *E.coli*. (**A**) Effects of photoreactivation on the survival of UV-irradiated *E.coli* SY32 (pRT2) cells carrying pGEX4T-2 vector only (circles) or pGEX-Xl64PR (triangles). After UV irradiation, the *E.coli* cells were kept in the dark (closed symbols) or illuminated with a fluorescent lamp (open symbols). (**B**) Coomassie brilliant blue stained SDS–polyacrylamide gel (10%). Lane 1, 30 µg total protein from cell extract of *E.coli* transformed with pGEX-Xl64PR (Fraction I); lane 2, 2 µg eluate from glutathione–Sepharose column (Fraction II); lane 3, 2µg eluate from glutathione–Sepharose column after thrombin cleavage (Fraction III); lane 4, 1 µg eluate from UV-irradiated DNA affinity column (Fraction IV); lane M, molecular weight marker. (**C**) Disappearance of the binding site for the (6–4)photoproduct-specific antibody in UV-irradiated DNA. Repair of UV damage in the photoreactivated DNA was quantified by ELISA using an antibody specific for (6–4)photoproduct (64M2, circles) or for CPDs (TDM2, triangles). UV-irradiated salmon sperm DNA was mixed with recombinant *Xenopus* (6–4)photolyase and kept in the dark (closed symbols) or illuminated with a fluorescent lamp for various periods (open symbols). Illuminated DNA without recombinant protein is also shown (dotted lines). (**D**) Restoration of (6–4)photoproduct by *Xenopus* (6–4)photolyase. 32 bp DNA containing a (6–4)photoproduct at the *Hpa*I site in its center was digested with *Hpa*I after photoreactivation with recombinant *Xenopus* (6–4)photolyase (lane 2), treated with buffer alone (lane 1) or recombinant protein in the dark (lane 3), illuminated with light without recombinant protein (lane 4).

and photocatalytic activity (Fig. 4B). The molar ratio of FAD released from *Xenopus* (6–4)photolyase relative to its apoprotein was 0.95 as calculated from the coefficients of the apoprotein ($\varepsilon_{280} = 1.30 \times 10^5$ $M^{-1}cm^{-1}$). The excitation coefficient for the *Xenopus* (6–4)photolyase apoprotein was calculated using the number of tryptophan (18; $\varepsilon_{280} = 5800$ $M^{-1}cm^{-1}$) and tyrosine (18; $\varepsilon_{280} = 1405$ $M^{-1}cm^{-1}$) residues determined from the DNA sequence of the *Xenopus* (6–4)photolyase gene. Together, these results show that *Xenopus* (6–4)photolyase binds FAD.

DISCUSSION

CPDs and (6–4)photoproducts are the two major classes of cytotoxic, mutagenic and carcinogenic photoproducts produced in DNA when cells are irradiated with UV light (2,3,5). These lesions are repaired by the nucleotide excision repair pathway, although CPDs are repaired less efficiently than (6–4)photoproduct. CPDs are most efficiently repaired by DNA photolyase (4). It has long been believed that CPDs are the only substrate for DNA photolyase. As a consequence, it has become common practice to expose UV-irradiated cells to photoreactivating light (350–450 nm) to study the effects of (6–4)photoproduct. Any residual mutagenic or cytotoxic effects remaining following photoreactivation are ascribed to (6–4)photoproduct (3). In contrast to the general belief that CPDs are the only substrate for photolyase, we discovered a new type of photolyase in *D.melanogaster* which catalyzed light-dependent repair of (6–4)photoproduct [(6–4)photolyase] (13). In this paper we have identified the *Xenopus* (6–4)photolyase gene. This is the first molecular description of a (6–4)photolyase gene in a vertebrate. An enzymatic activity of (6–4)photolyase has also been detected in the rattlesnake and a higher plant (14,15), indicating that (6–4)photolyase might be widely distributed among present organisms. Thus, interpretations of the effects of photoreactivation on UV-irradiated cells reported previously should be reconsidered. In frog cells (ICR 2A) (6–4)photoproduct was removed rapidly from DNA of UV-irradiated cells following photoreactivation (26). This might show photoreactivation of (6–4)photoproduct in ICR 2A cells, although it was interpreted

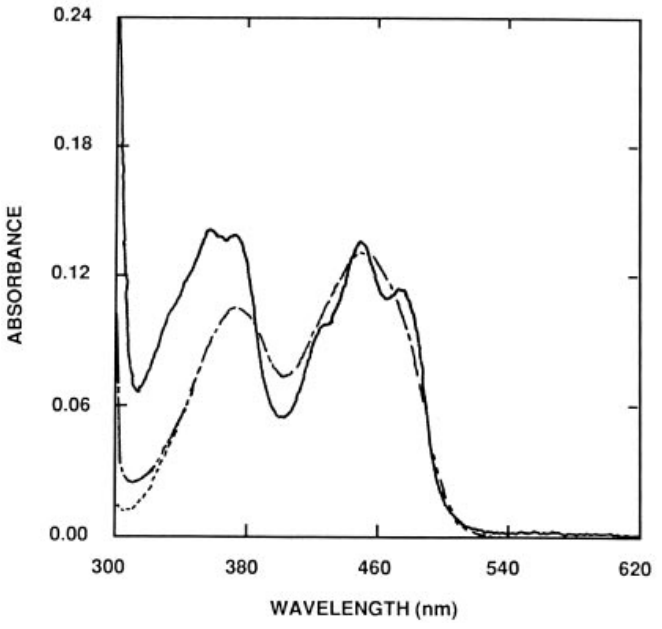

Figure 3. Comparison of absorption spectra of native *Xenopus* (6–4)photolyase prepared from *E.coli* (—), the chromophore released from *Xenopus* (6–4)photolyase by acid denaturation (– – –) and authentic FAD at pH 3 (------).

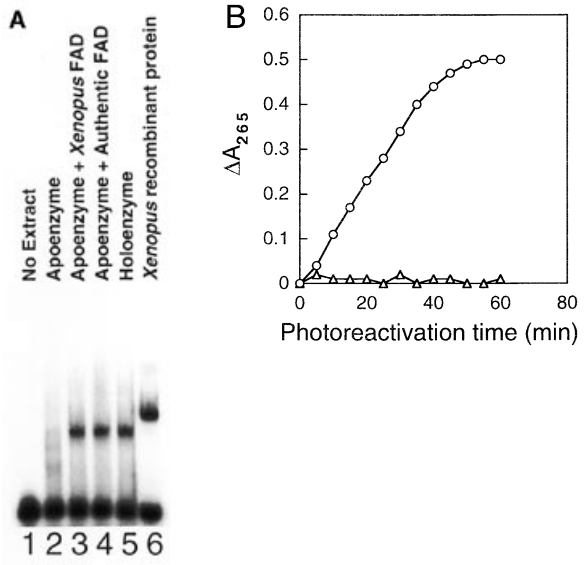

Figure 4. Reconstitution of *E.coli* CPD photolyase activity with FAD isolated from *Xenopus* (6–4)photolyase. (**A**) Gel shift analysis showing reconstitution of *E.coli* CPD photolyase activity with apoenzyme and FAD isolated from *Xenopus* (6–4)photolyase. UV-irradiated TC-3 DNA probe (16) was used for gel shift assay with *E.coli* photolyase apoenzyme (lane 2), *E.coli* photolyase apoenzyme reconstituted with FAD isolated from *Xenopus* (6–4)photolyase (lane 3) or with authentic FAD (lane 4), *E.coli* photolyase holoenzyme (lane 5) or *Xenopus* (6–4)photolyase (lane 6). (**B**) CPD photolyase activity of reconstituted *E.coli* photolyase. *Escherichia coli* photolyase apoenzyme (triangles) or enzyme reconstituted with FAD isolated from *Xenopus* (6–4)photolyase (circles) was assayed for photolyase activity in the presence of CPD-containing oligo(dT)$_{20}$ as substrate. ΔA_{265}, change in absorbance at 265 nm.

that the removal of CPDs following photoreactivation led to an increase in the capability for excision of (6–4)photoproduct (26).

We have demonstrated that the (6–4)photolyase is a flavoprotein, similar to other members of the DNA photolyase/blue light photoreceptor family. Each member of this family utilizes light energy through FAD in various reduced forms. In CPD photolyase, $FADH^-$ is the active form which donates an electron to the CPD, resulting in splitting of the cyclobutane ring (7). In the blue light photoreceptor, oscillation of FAD between its different redox states determines the response wavelength for each plant cell (12). Photoreduction of purified *Xenopus* (6–4)photolyase led to activation of repair activity (data not shown), indicating that reduced FAD is the active form and (6–4)photoproduct is repaired by electron donation, as is the case for CPD. A possible pathway for repair by (6–4)photolyase was proposed previously (16), in which binding of (6–4)photolyase to DNA was suggested to thermally convert the (6–4)photoproduct to its oxetane intermediate and then electron transfer from excited FAD to the intermediate restored the original form. Our results are consistent with this model. Complete understanding of the repair mechanism must await further characterization of (6–4)photolyase.

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